REMARKS

I. Status

Claims 1-9 were pending and have examined by the Examiner. By this Amendment, claims 2-3 and 5-13 are presented for examination.

Regarding the specification, the foregoing amendments are made to insert sequence identification number identifiers as requested by the Examiner.

II. Objections to the Specification

In response to the Examiner's objection to the abstract of the disclosure, the abstract of the disclosure has been placed on a separate sheet. No new matter has been added.

Furthermore, with respect to the Examiner's objection to the Figures, a copy of Figures 1-6 which include English language translation of the legends are attached hereto.

In response to the objections to the specification as lacking certain sequence listings, applicants submit herewith amendments to the specifications adding sequence listing number identifiers. Applicants also are filing herewith a paper copy and a computer readable copy of the supplemental sequence listing and a statement in support thereof as well as a copy of the Notice to Comply. As a point of clarification, applicants note that on the page following the cover page of the Notice to Comply there appears to be in error in the statement that page 69, line 26, page 70, line 4, page 79, line 25, page 85, line 3 and 4 and figure 7 contain sequences.

III. Response to Rejections

A. Indefiniteness Rejections

The Office Action has rejected claims 1-9, under 35 U.S.C. § 112, as being indefinite for failing to particularly point out and distinctly claim the subject matter.

In response to the objection of the claims regarding the use of "a" and "the", applicants have amended the claims to use "said" to indicate a reference to an antecedent basis and have replaced "the" when an antecedent basis reference is not intended.

Furthermore, the amended claims obviate the other indefiniteness rejections.

B. Obviousness Rejections

Applicants note that with regard to the state of the art regarding the claimed invention:

- the production of transgenic animals, such as mice to express a chimeric protein, was a conventional operation for a skilled artisan in the art as shown, for example, by the BUJARD et al. U.S. patent no. 5,866,755 ("the BUJARD patent"), cited by the Examiner in the Form 892 attached to the instant Office Action, and

- on the other hand, the properties of the polypeptide nectin-1[1] (also known as HveC, HigR, or PRR1) as a functional receptor of the alphaherpesviruses and, in particular, the viruses HSV, PRV and BHV-1 were also known to the skilled artisan.

In this context, the problem existing in the art that is solved by the claimed invention consists of producing lines of mammals and, for example, in transgenic porcine and bovine species rendered resistant to infection by specific alphaherpesviruses (*e.g.*, in the case of transgenic porcine and bovine species, PRV or BHV-1) for which polypeptide nectin-1 is a functional receptor.

To overcome this problem, the inventors had to select this specific functional receptor (from the different input mediators of the alphaherpesvirus in the cell described in the literature) and to use it in transgenesis to obtain lines of mammals resistant to these

[1] For the sake of convenience, applicants use the nomenclature "nectin-1" in this response but note that HveC and other terms are also used in the art to denote the nectin-1 polypeptide.

alphaherpesviruses, by turning one of its properties to their advantage. Accordingly, this specific functional receptor nectin-1, which is present on the surface of many types of cells, is used outside its physiological context.

Furthermore, the claimed invention is further patentable in reciting an extracellular domain that is truncated (that is, "a part thereof") from the form normally present.

Therefore, according to the invention, it is set forth that one introduces into the genome of a mammal, in addition to the molecule of the polypeptide nectin-1, which is normally present, an additional form of this molecule, which additional molecule does not play its usual physiological role and may be expressed in significant supraphysiological quantities, in soluble form.

This molecule, whether circulating or on the surface of the cells, acts as a decoy for the non-productive binding of the alphaherpesvirus; that is, binding not followed by the entry of an infectious virion into the cell.

In other words, according to the invention claimed herein, superimposed onto the functional receptor normally present, an inactive form, which may be in significant quantities, of this receptor is used in order to block the process by which the alphaherpesvirus enters the cell.

Accordingly, the claimed invention of producing lines of transgenic mammals rendered resistant to infection by an alphaherpesvirus is non-obvious over the prior art cited by the Examiner.

In further support of the non-obviousness of the claimed invention over the art cited by the Examiner, applicants direct the Examiner to the article by Ono, *et al.* "Transgenic mice expressing a soluble form of porcine nectin-1/herpesvirus entry mediator C as a model for pseudorabies-resistant livestock", published in the journal PNAS vol. 101, no. 46 (2004), attached hereto; showing that researchers have attempted to produce lines of transgenic animals resistant to infection by alphaherpesviruses as early as the late 1980s.

Previously, such research consisted of attempting to introduce a transgene into the genome of such animals, allowing the expression of a modified form of the virus. These attempts were unsuccessful.

The inventors, on the other hand, conceived of taking a totally different path from the previous research, which consisted of not using modified components of the virus to alter the susceptibility of the host, but instead using a domain from the host, known for its interaction with the viral cycle (in this case, one of the molecules that allows the virion to enter the cell) and expressing the domain in an entopic form, such as to render the infection non-productive, by turning the receptor-virus interaction away from its usual result.

Regarding the art cited by the Examiner as grounds for instant rejection, the Examiner states that claims 1-5 are obvious in view of the FIUME, *et al.* U.S. patent no 6,469,155 ("the FIUME patent"), considered together with the BUJARD patent.

In response, applicants submit that, as noted by the Examiner, the BUJARD patent discloses that the production of transgenic animals such as mice expressing a chimeric protein constitutes a conventional operation in the art. As set forth above, Applicants concede that this was known in the art; however, this aspect of the art does not render claims 1-5 obvious. The BUJARD patent, therefore, does not render claims 1-5 obvious.

With regard to the FIUME patent, as noted by the Examiner, this patent discloses that the polypeptide nectin-1 (referred to as HveC in the FIUME patent according to the Examiner's rejection) is involved in the propagation of certain alphaherpesviruses in the cell. As set forth above, Applicants concede that the functional receptor of the alphaherpesvirus was known in the art. Furthermore, the patent mentions that the domain V of the polypeptide nectin-1 is a ligand of the alphaherpesvirus, and the patent proposes using this domain polypeptide as a structural basis for antiviral molecules likely to recognize alphaherpesviruses.

In other words, the patent suggests using the domain V of nectin-1 bound to another non-stipulated molecule, to obtain external prophylactic or therapeutic agents that may be

used in the field of human medicine locally against infections by the alphaherpesvirus and, in particular, for drugs or vaccines.

Furthermore, according to the FIUME patent, another use of the protein nectin-1 is proposed, namely, the development and use of an animal model to test active therapeutic or prophylactic agents against infections by HSV or BHV-1 in the form of transgenic mice particularly sensitive to such infections.

Nevertheless, it is proposed to introduce into the genome of these transgenic mice a coding transgene not only for the extracellular domain of the nectin-1 but for the entire molecule in order, of course, to render these mice sensitive to viruses to which they are not inherently sensitive or relatively insensitive (HSV or BHV-1).

The rationale behind this use is that the introduction of a viral receptor, such as the protein nectin-1 in full, creates an opening for the virus to enter the cell, thereby increasing or creating the sensitivity of transgenic animals to the virus.

This potential effect is described in the FIUME patent for cultured cells *in vitro* (the expression of the nectin-1 allows for the multiplication of the virus in culture, which also contributes to qualifying its roles as a viral receptor).

It is essential to note that, for transgenic mice used to test the potential pharmacological efficacy of molecules that are candidates for prophylactic or therapeutic use for pathologies due to infection by an alphaherpesvirus, it is essential that such mice models are particularly sensitive to such infections and develop clear, marked and easy to measure symptoms in response thereto.

There is, of course, no point in rendering an animal model resistant to a viral infection to then test reagents on this model, which could provide protection against the pathological conditions associated with this infection. In this case, the pathological conditions associated with the infection would not exist due to the resistance to the infection and could not therefore be treated by the molecules to be tested.

Thereafter, the FIUME patent does not teach or suggest the skilled artisan to produce a mammal rendered resistant to alphaherpesvirus infection by inducing into the genome of mammals (*e.g.*, into porcine or bovine species) a coding transgene for the extracellular domain of nectin-1. This is particularly true for the production of lines of such transgenic mammals rendered constitutionally resistant to infection by specific alphaherpesviruses (*e.g.*, PRV or BHV-1), the polypeptide of which constitutes one of the functional receptors.

Indeed, even if it is assumed that the FIUME patent would have motivated the skilled artisan to produce such lines of transgenic mammals according to a mice animal model of the infection for HSV and using the protein nectin-1 in full, the skilled artisan would normally expect such mammals to be more sensitive to infection by an alphaherpesvirus than their non transgenic homologous species, due to the facilitation of the entry of the virus.

Only a later interpretation implying prior, therefore inadmissible, knowledge of the invention, would suggest the opposite result. That is, that such transgenic animals would be, on the contrary, resistant and protected against this virus.

Accordingly, the FIUME patent, either alone or in combination with the BUJARD patent, does not render obvious rejected claims 1-5.

C. Written Description Rejections

The Examiner alleges that claims 1-9 fail to comply with the written description requirements.

In response to the Examiner's rejection, applicants submit the following.

The polypeptide sequence of nectin-1 is remarkably well conserved between species of mammals; for example, 97% of amino acids are common to the protein nectin-1 expressed by porcine species and that expressed by bovine species, which provides a reasonable basis for assuming a significant structural and functional identity between the two species.

Furthermore, Applicants respectfully submit that grounds for the rejection do not account for the following: (1) the names HveC and nectin-1 refer to the same polypeptide and

(2) Example 2 of the instant specification refers to resistance to the virus PRV of transgenic mice, into which has been introduced a coding transgene for a chimeric protein consisting of the extracellular domain of the porcine receptor HveC (and not the murine receptor HVEM) and crystallizable fragment Fc of the human immunoglobulin IgG-1.

In view of the foregoing, applicants respectfully request reconsideration and withdrawal of the written description rejection.

D. Enablement Rejections

The Examiner alleges that claims 1-9 are non-enabled. However, the Examiner submits on page 12 of the Office Action that claims 1-5 are enabled for transgenic mice. Applicants respectfully respond as follows.

Contrary to the position of the Examiner, the invention of claims is sufficiently taught by the instant specification.

According to Example 1, it is shown that transgenic mice expressing a chimeric protein consisting of the extracellular domain of the murine polypeptide HVEM and the crystallizable fragment Fc of the human immunoglobulin IgG-1, are protected against the virus HSV-1, the polypeptide HVEM of which constitutes a functional receptor whereas they are not protected against the virus PRV, the protein HVEM of which does not constitute a functional receptor.

According to Example 2, it is shown that transgenic mice expressing a chimeric protein consisting of the extracellular domain of the porcine polypeptide nectin-1 which is a functional receptor of the virus PRV and of the crystallizable fragment Fc of the human immunoglobulin IgG-1 are protected against the virus PRV.

According to Example 3, it is shown that Vero lines and therefore simian cells, transformed by plasmids expressing chimeric proteins from the extracellular domain of the porcine polypeptide nectin-1 and the crystallizable fragment of the human immunoglobulin Ig are resistant to infection by the virus PRV and BHV-1, the polypeptide nectin-1 of which constitutes a functional receptor.

Finally, according to Example 4, it is shown that Vero line cells transformed by plasmids expressing chimeric proteins created from the extracellular domain of the porcine polypeptide nectin-1 and the crystallizable fragment of the porcine immunoglobulin IgG are also resistant to infection by the virus BHV-1 and PRV, the polypeptide HveC of which constitutes a functional receptor.

Thus, the specification shows that by expressing the functional receptor corresponding to a virus, the inventors obtained resistance specific to this virus, whilst protection is not obtained against a virus when the expressed polypeptide does not constitute a functional receptor, whatever species of transgenic mammal is concerned.

In particular, it is irrelevant that during in-vivo experiments, the inventors only had access to a mouse model and not a porcine or bovine model, in that a mouse model is much easier to use in experiments that experiments carried out on porcine models have shown that there is indeed little difference between the species.

With respect to the Examiner's citation of the publication MULLINS, *et al.*, applicants note that this publication has a date of 1996 which does not reflect the state of the art at the time of filing of the invention.

Furthermore, the fact that the efficacy of the system has shown in three different species (mice, monkeys and pigs) is, applicants submit, sufficient to substantiate that the claims are enabled.

Moreover, applicants respectfully traverse the Examiner's arguments, based on the publication MACHATY, *et al.*, which according to the Examiner relates to the production of transgenic animals by pronuclear microinjection of transgenes and according to the Examiner discloses that this process is random, due to the position of the insertion of the transgene. First, applicants submit that the specification directs the skilled artisan to, for example, the homologous recombination technique which does not have such insertion effects.

Moreover, even in the case of pronuclear microinjection the effect of the allegedly random integration does substantiate the examiner's alleged finding that unreasonable

experimentation would be required to make and use the invention of claims 1-9. In pronuclear microinjection the skilled artisan does not use only one founding animal and a single line, but instead several lines are produced. With the use of several lines, it can be shown, without undue experimentation, whether there is a correlation between the expression of the chimeric protein and the effect obtained

Enablement of transgenic mammals expressing a chimeric protein containing nectin-1

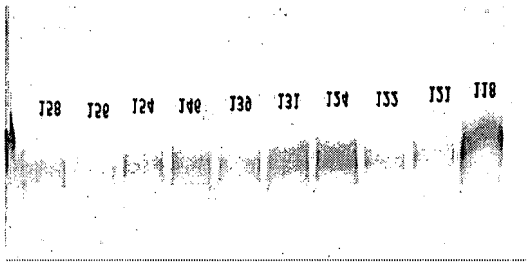
Moreover, contrary to rejection, the specification describes transgenic mice expressing a chimeric protein containing the extracellular domain of the porcine polypeptide nectin-1, which is a functional receptor of the virus PRV and the crystallizable fragment Fc of the human immunoglobulin IgG-1.

Furthermore, transgenic pigs have now been obtained according to the details of the specification.

Seven lines of transgenic pigs have been obtained in accordance with the description of the application by pronuclear micro injection of the fragment SnaBI/SalI of the plasmid Pcxn2/pHveCIg described in example 2 of the application and which allow for the expression in all cell types, of a chimeric protein comprising the extracellular domain (VCC) of the nectin-1 fused with the fragment Fc of the human immunoglobulin IgG, under the control of the promoter AG, according to the conditions described in example 2 of the application and the ONO et al., article cited above.

The expression of the chimeric protein has been checked in the serum of the founding animals FO by Western Blot, using an antibody, antifragment Fc, human IgG and which reveal the presence of a specific protein of approximately 60kDa for an expected molecular

weight of 61 kDa.



The resistance to the virus PRV of these lines of pigs has been documented by tests in vitro:

on the serum of the founding animals FO:

Sero-neutralization of 100 DECP 50 PRV / 50 µl serum / 2 wells 96 serum

Id Serum	Neutralization (+/- = cytopathic effect observed on	Dilution of serum
F0/118	+/-	1/2
F0/122	+/-	1/2
F0/124	+	1/1
F0/131	+/-	1/1
F0/156	+/-	1/1
F0/158	+	1/1
Negative control:	-	-
Negative control:	-	-

on embryonic fibroblasts isolated from transgenic fetuses taken from these lines, as described in example 2.

Number and diameter of the plaques observed, average of 2 boxes of 60 mm, 25 DECP/box, plaques counted in an agarose solution of 0.5 % for 3 days p.i. after dyeing with hexamethyl pararosaniline chloride.

Id. Fibroblasts	Diameter of plaques	Number of
Transgenic	2.56	53.0
Controls	2.67	98.0

A reduction in the number of plaques (half the number of plaques) was therefore observed for embryonic fibroblasts taken from transgenic pigs.

In view of the forgoing, applicants respectfully request reconsideration and withdrawal of the enablement rejection.

Conclusions

Applicants believe that the present application is now in condition for allowance. Favorable reconsideration of the application as amended is respectfully requested.

The Examiner is invited to contact the undersigned by telephone if it is felt that a telephone interview would advance the prosecution of the present application.

Respectfully submitted,

Date 4/30/07 By _____

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Should additional fees be necessary in connection with the filing of this paper, or if a petition for extension of time is required for timely acceptance of same, the Commissioner is hereby authorized to charge Deposit Account No 50-3380 referencing Attorney Docket No. HER0071 for any such fees; and applicants hereby petition for any needed extension of time.

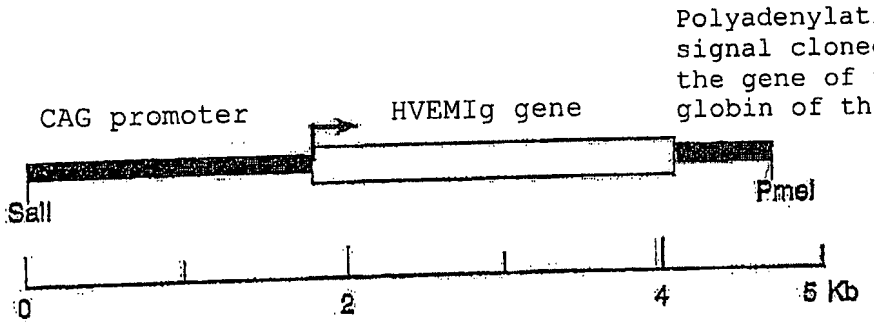

FIGURE 1

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FIGURE 2

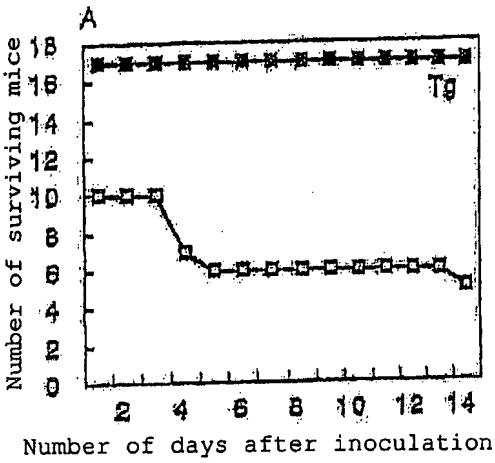
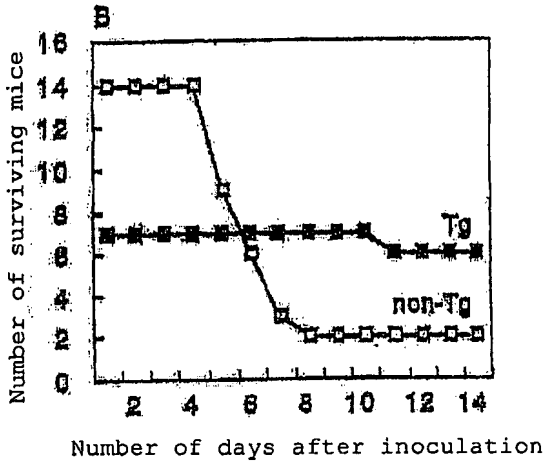


FIGURE 3



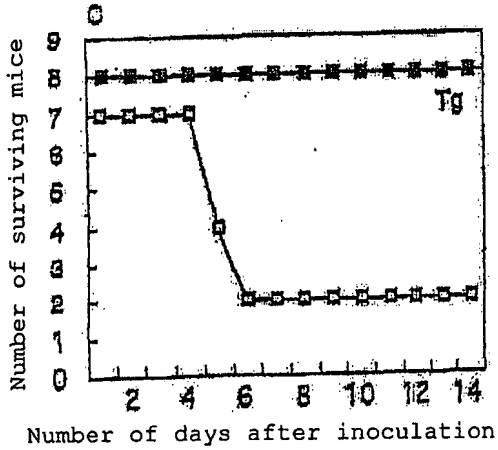


FIGURE 4

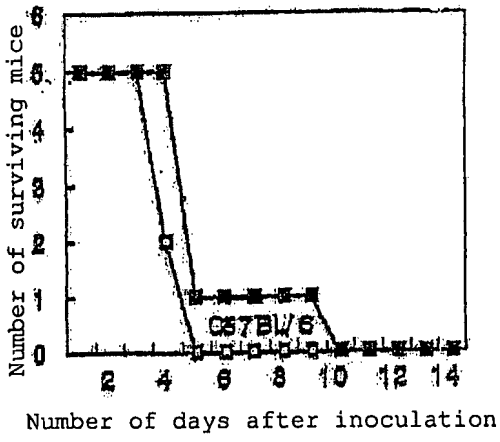
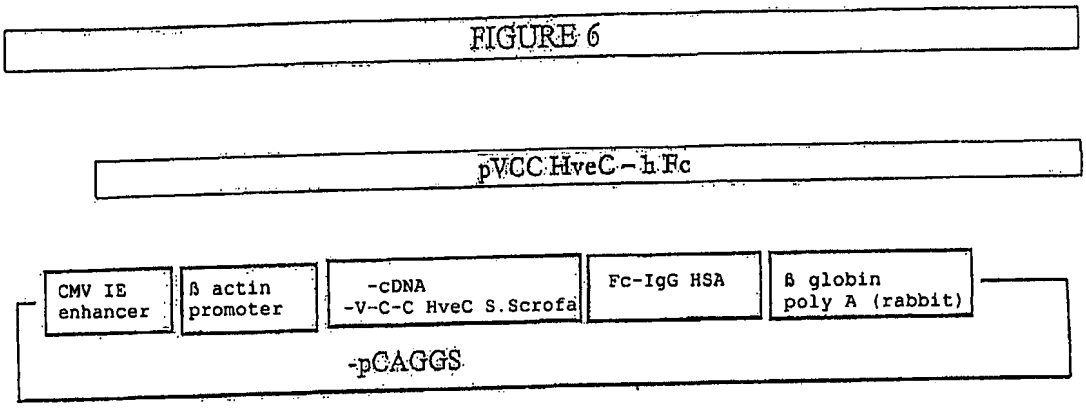
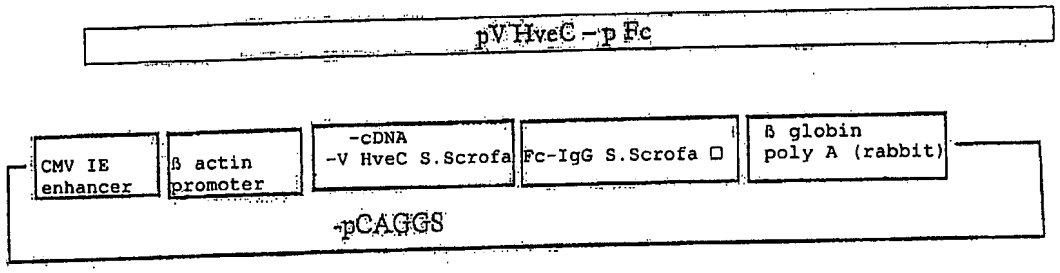


FIGURE 5

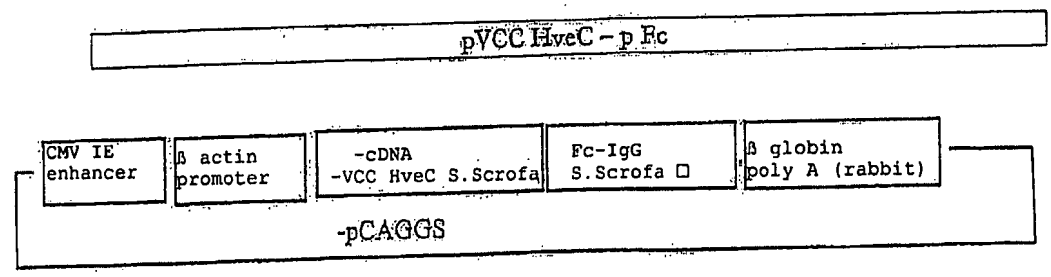
FIGURE 6



1.1.1. V domain of the porcine nectin-1 (HveC) and crystallisable fragment of pig immunoglobulin G1



1.1.2. Extracellular domain of the porcine nectin-1 (HveC) and crystallisable fragment of pig immunoglobulin G1



Transgenic mice expressing a soluble form of porcine nectin-1/herpesvirus entry mediator C as a model for pseudorabies-resistant livestock

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Edited by Patricia G. Spear, Northwestern University, Chicago, IL, and approved October 1, 2004 (received for review August 9, 2004)

An approach to genetically engineered resistance to pseudorabies virus (PRV) infection was examined by using a transgene encoding a soluble form of nectin-1, also known as herpesvirus entry mediator C. Nectin-1 is an α-herpesvirus receptor that binds to virion glycoprotein D. Nectin-1 mediates entry of PRV, herpes simplex virus types 1 and 2, and bovine herpesvirus type 1. To assess the antiviral potential of an ectopic expression of the nectin-1 ectodomain *in vivo*, six transgenic mouse lines expressing a soluble form of nectin-1, consisting of an extracellular domain of porcine nectin-1 and the Fc portion of human IgG1, were generated. All of the transgenic mouse lines showed nearly complete resistance to PRV infection by means of both i.p. and intranasal routes. These results suggest that the introduction into farm animals of a transgene encoding a soluble form of nectin-1 would offer a potent biological approach to generating α-herpesvirus-resistant livestock.

Altering host susceptibility to viral infections through vaccination is a widespread strategy used to reduce associated pathologies and virus shedding. Viral disease control in farm animals also offers significant routes to improved host resistance through selection for existing resistant alleles or expression of transgenes, the products of which interfere with the viral replication cycle. Up to now, the development of disease-resistant farm animals by gene transfer has been almost nonexistent.

Pseudorabies virus (PRV), a representative member of the α-herpesvirius subfamily, causes lethal encephalitis in piglets, acute respiratory syndrome in growing pigs, abortion and infertility in breeding sows, and latent infection in surviving pigs. PRV infection is a major economic risk in the swine industry worldwide. In general, vaccination strategies alone usually suppress manifestation of the disease but do not stamp out viral infection from a population. Dominant-negative mutants of viral proteins have been demonstrated in cell culture systems as potent inhibitors of herpesvirus replication and proposed as a disease control strategy termed "intracellular immunization" (1). Intracellular immunization against herpesvirus infection *in vivo* was obtained by using mutated forms of the virus transcription factors (2, 3). Unfortunately, it appeared that these transgenes might exert adverse side effects, such as retarded growth, by hitting unknown host targets and thus illustrating a tight level of host–pathogen interactions during all steps of development. As an alternative to these previous strategies, we proposed to alter known host molecular components of the viral infection cycle.

We have set our sights on inhibition of viral entry to induce protection of the animals against PRV infection. Binding of α-herpesviruses to cells occurs primarily through an interaction of glycoprotein C and/or glycoprotein B with cell-surface heparan sulfate (4–7), whereas fusion between the virion envelope and cell membrane requires glycoproteins B, D, H, and L (8–11). Five α-herpesvirus receptors have been identified: herpesvirus entry mediator (Hve)A (HVEM), HveB (nectin-2), HveC (nectin-1), HveD (CD155), and 3-*O*-sulfated heparan sulfate (12–16). Recently, we reported that expression of a soluble form of HVEM in transgenic mice enhanced resistance to herpes simplex virus type 1 (HSV-1) infection (17). However, HVEM has no entry activity for PRV or bovine herpesvirus type-1 (12). In contrast, nectin-1 has the broadest specificity for mediating α-herpesvirus entry and is present in a broad range of tissues and cells (13, 14). The nectin family has the same Ig-like domain structures (18–22) (Fig. 1*A*). We demonstrated that transformed cell lines expressing a soluble form of porcine nectin-1 showed significant resistance to PRV infection (23). This *in vitro* model system provided a possible basis for the development of livestock with enhanced resistance to pseudorabies.

Here we demonstrate that transgenic mouse lines expressing a soluble form of porcine nectin-1 were highly resistant to PRV infection without any side effects. If this approach can be applied to farm animals, it could have a significant impact on animal production industries worldwide.

Materials and Methods

Generation of Transgenic Mice. The cDNA of the extracellular domain of porcine nectin-1 was amplified as described by Milne *et al.* (24). The cDNA was inserted into the *Xho*I and *Bam*HI sites of a plasmid carrying IgG1-Fc DNA (25). The chimeric gene was inserted into an *Xho*I site of pCXN2 vector (26) having a *Sal*I site instead of *Hin*dIII site. The *Sal*I transgene fragment (Fig. 1*A*) containing the CAG promoter (cytomegalovirus IE enhancer and chicken β-actin promoter), the PHveCIg gene, and the rabbit β-globin polyA signal was isolated and purified. The DNA fragment was microinjected into C57BL/6 mouse eggs to generate transgenic mice. Transgenic founders were identified by PCR with genomic DNA isolated from mouse tail (27) and the specific primers (PHveC-F, 5′-GGACCCCTCGAGCGCCATGGCT-3′; 3IG1, 5′-TGC CCT GGA CTG GGG CTG CAT AG-3′). The transgenic founders were crossed with C57BL/6 mice. Heterozygous F_1 transgenic mice were crossed with C57BL/6 mice, and their offspring (F_2) was used for experimental infections. All mice were maintained in the animal facilities at our institute and treated according to the Laboratory Animal Control Guidelines of our institute, which are similar to

This paper was submitted directly (Track II) to the PNAS office.

Abbreviations: Hve, herpesvirus entry mediator; HVEM, HveA; PRV, pseudorabies virus; HSV-1, herpes simplex virus 1; LAT, latency-associated transcript.

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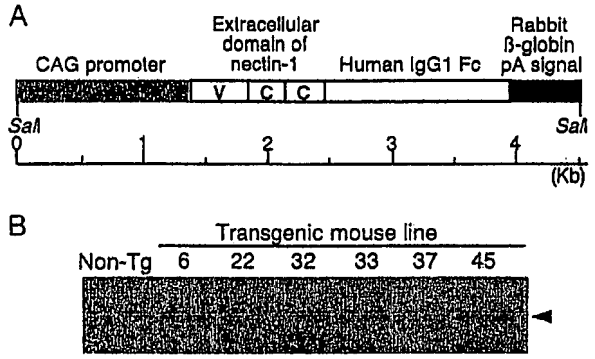

Fig. 1. Generation of transgenic mice expressing PHveCIg. (*A*) Schematic representation of the transgene. The PHveCIg gene is under the control of the CAG promoter. The extracellular domain of nectin-1 consists of one V domain and two C domains. Cleavage sites for restriction enzyme *SalI* are indicated. (*B*) Western blot analysis of sera from the transgenic (Tg) mice. The arrow indicates the position of detected PHveCIg.

those of the National Institutes of Health American Association of Laboratory Animal Control.

Statistical Analysis. Means test comparisons were done by using ANOVA (litter size) and general linear modeling (body weights) procedures from SAS 8.12 software. (SAS Institute, Cary, NC). Means comparisons were done by using pairwise *t* tests.

Analysis of Transgene Expression. A reference PHveCIg protein sample was purified from a supernatant of the transformed Vero cell line (C-A6) expressing PHveCIg (23). To measure PHveCIg concentrations in sera of the transgenic mice, a competitive ELISA system using a rabbit anti-human nectin-1 antibody (28) was established as described in ref. 17. Western blotting with 1 μl of each serum of the transgenic mice and histopathological procedure was performed as described in ref. 17. The rehydrated sections were immunostained by the indirect immunoperoxidase technique with biotinylated anti-human IgG and avidin-horseradish peroxidase detection reagent.

Virus Infection in Mice. PRV strains YS-81, Kojnock, Chiba-03, a new field isolate from Japan (developed in 2003), and HSV-1 strain VR-3 were used for experimental infections. The LD_{50} of each virus strain were titrated on C57BL/6 mice. The mice at 6–8 weeks of age were infected i.p. with 200 μl of DMEM containing 20 LD_{50} of PRV strain YS-81 in Sapporo, Japan, or strain Kojnock in Paris. Experimental infection with HSV-1 was also performed as described above. Intranasal PRV infection was performed with 5 μl of DMEM containing 10 LD_{50} of PRV strain YS-81 or strain Chiba-03 under anesthesia. Survival of mice and signs of disease were recorded for 14 days. Anti-PRV antibodies in sera of surviving mice at least 1 month after the virus inoculation were measured by ELISA, with disrupted-purified PRV as the viral antigen (3).

Detection of the Virus DNA in Trigeminal Ganglia by PCR. Mice surviving intranasal infections were killed by decapitation at least 1 month after the virus inoculation, and trigeminal ganglia were immediately removed and frozen in liquid nitrogen. As a control experiment, transgenic mice and nontransgenic littermates were infected with PRV strain Begonia, an attenuated vaccine strain deleted for glycoprotein E and thymidine kinase genes (Intervet International, Boxmeer, The Netherlands). Genomic DNA was isolated from trigeminal ganglia and screened for PRV latency-associated transcript (LAT) sequences. PRV DNA was detected by PCR analysis with the specific primers for the PRV LAT gene (LAT-F, 5′-GAGGAGGAGGACACGA-3′; LAT-R, 5′-TCCAGCTCCGGCACCAAGT-3′). PCR for the LAT gene was carried out as described in ref. 29. Digoxigenin-labeled DNA probes for detection of the virus DNA were derived from pG/*Kpn*I-E (30) by using the specific primers described above and a PCR digoxigenin probe synthesis kit (Roche, Gipf-Oberfrick, Switzerland). Southern blotting of the PCR products was performed as described in ref. 3.

Table 1. Characteristics of transgenic mouse lines expressing PHveCIg

Line	Copy no.	PHveCIg in serum, μg/ml	Body weight, g	Litter size
6	1	1,820.5 ± 188.3	16.2 ± 1.8[a] (4)	8.0 ± 1.9[d]
22	4	258.0 ± 100.5	18.9 ± 2.3[b] (3)	7.4 ± 1.9[d]
32	20	742.9 ± 47.9	18.3 ± 1.6[b,c] (8)	6.0 ± 1.0[d]
33	3	1,283.0 ± 370.8	17.1 ± 1.2[b,c] (7)	7.6 ± 1.7[d]
37	50	5.0 ± 1.9	17.5 ± 1.6[a,b,c] (6)	7.2 ± 1.3[d]
45	2	1,180.1 ± 279.9	18.2 ± 1.6[b,c] (8)	3.8 ± 2.2
C57BL/6	0	1.2 ± 0.6	17.4 ± 1.5[a,b,c] (8)	6.2 ± 1.3[d]

Copy number was estimated by Southern blot analysis, and the amount of PHveCIg in serum was measured by competitive ELISA with at least three transgenic offspring. Shown is the body weight of 8-week-old female mice, with the number of transgenic mice tested shown in parentheses. Litter size was measured for five litters. Means sharing the same superscript letter are not significantly different from each other at threshold $P < 0.05$.

Virus Infection in Cultured Cells. Transgenic and nontransgenic embryonic fibroblasts were prepared as described in ref. 17. Immunofluorescence assay with FITC-labeled goat anti-human IgG (Fc), Western blotting, and the plaque assay were performed as described in ref. 23.

Results

Characterization of Transgenic Mice. To assess the antiviral potential of nectin-1 *in vivo*, we generated six transgenic mouse lines expressing a soluble form of porcine nectin-1 (PHveCIg) consisting of an extracellular domain of porcine nectin-1 and the Fc portion of human IgG1. In these transgenic mice, the PHveCIg gene was under the control of the CAG promoter (Fig. 1*A*), which allows an expression in all cell types (26). Southern blot analysis performed on genomic DNA from these mice showed that the transgenic mouse lines harbored different transgene copy numbers (1 copy for line 6, 4 copies for line 22, 20 copies for line 32, 3 copies for line 33, 50 copies for line 37, and 2 copies for line 45) (Table 1). By using a rabbit anti-human nectin-1 antibody (28), Western blot analysis revealed that the transgene was expressed in all of the six transgenic mouse lines (Fig. 1*B*). As shown in Table 1, PHveCIg concentrations in serum samples from the different mouse lines were different in a large extent ranging from 5.0 ± 1.9 to 1,820.5 ± 188.3 μg/ml.

One major concern with the transgenesis approach is possible adverse side effects generated by the transgene. However, no significant abnormalities in the transgenic mice were observed. They developed normally, and there were no significant differences in body weight between transgenic mice and control mice (Table 1). Moreover, the transgenic mouse lines had a normal capacity to reproduce and lactate, except for line 45 (Table 1). Histological studies with hematoxylin and eosin staining with tissue sections, including main organs, from the transgenic and the nontransgenic littermate mice of each transgenic mouse line at 7 weeks of age were performed to examine abnormalities of phenotypic appearance in the transgenic organs. No difference between the transgenic and the littermate mice was observed,

Table 2. Survival rates of mice after the challenge with α-herpesviruses

Line	Transgenic mice, %	Control littermates, %	Virus	Dose	Route	Location
6	100 (5/5)	14 (2/14)	PRV YS-81	20 LD_{50}	i.p.	Sapporo
22	100 (12/12)	0 (0/12)	PRV YS-81	20 LD_{50}	i.p.	Sapporo
32	100 (10/10)	14 (1/7)	PRV YS-81	20 LD_{50}	i.p.	Sapporo
33	86 (6/7)	0 (0/9)	PRV YS-81	20 LD_{50}	i.p.	Sapporo
37	100 (10/10)	25 (1/4)	PRV YS-81	20 LD_{50}	i.p.	Sapporo
45	100 (6/6)	8 (1/12)	PRV YS-81	20 LD_{50}	i.p.	Sapporo
22	100 (7/7)	0 (0/1)	PRV Kojnock	20 LD_{50}	i.p.	Paris
32	100 (7/7)	14 (1/7)	PRV Kojnock	20 LD_{50}	i.p.	Paris
6	100 (6/6)	14 (1/7)	HSV-1 VR-3	20 LD_{50}	i.p.	Sapporo
6	50 (9/18)	0 (0/9)	PRV YS-81	10 LD_{50}	i.n.	Sapporo
22	78 (18/23)	10 (2/21)	PRV YS-81	10 LD_{50}	i.n.	Sapporo
32	61 (11/18)	8 (1/12)	PRV YS-81	10 LD_{50}	i.n.	Sapporo
33	65 (11/17)	12 (1/8)	PRV YS-81	10 LD_{50}	i.n.	Sapporo
37	90 (9/10)	8 (1/12)	PRV YS-81	10 LD_{50}	i.n.	Sapporo
45	100 (9/9)	10 (1/10)	PRV YS-81	10 LD_{50}	i.n.	Sapporo
37	100 (12/12)	10 (1/10)	PRV Chiba-03	10 LD_{50}	i.n.	Sapporo

Deaths were recorded daily until the termination of the experiments at day 14. Shown are the survival percentages for transgenic mice and their littermates, which were used as controls. The values in parentheses are the ratios of surviving mice to the number of mice tested. Locations where experimental infection were performed are also shown. i.n., intranasal.

and each tissue section observed was scored as normal for the tissue and the age considered. In a rotarod test (31), the motor coordination and balance of the transgenic mice was not significantly different from that of control mice (data not shown).

Resistance to i.p. Challenge with PRV. To find out whether the transgenic mice expressing PHveCIg were protected against PRV infection, 20 LD_{50} of PRV strain YS-81 was i.p. inoculated into transgenic mice and their nontransgenic littermates. The survival data (Table 2) demonstrate that the transgenic mice from all lines showed remarkable resistance to PRV infection. Only one transgenic mouse, from line 33, died after infection. In contrast, >90% of their littermates died within 14 days (Table 2). Specific antibodies to PRV were not detected in the surviving i.p. inoculated transgenic mice tested by ELISA (data not shown), indicating that the mice were not infected with PRV. To confirm this resistance, experimental infections were repeated in the French laboratories with 20 LD_{50} of a different strain of PRV, strain Kojnock. All transgenic mice of lines 22 and 32 that were tested survived, whereas almost all of their littermates died (Table 2). Furthermore, it is known that porcine nectin-1 is a glycoprotein D receptor for HSV-1 (24). To find out whether the transgenic mice expressing PHveCIg were protected against HSV-1 infection, 20 LD_{50} of HSV-1 strain VR-3 was i.p. inoculated into the transgenic mice of line 6 and their littermates. All transgenic mice survived, and six of seven nontransgenic littermates died within 14 days after HSV-1 infection (Table 2).

Resistance to Intranasal Challenge with PRV. Because PRV usually enters the body in pigs by infection of mucosal epithelium, intranasal challenges with PRV were performed. Ten LD_{50} of YS-81 strain was intranasaly inoculated into the transgenic mice and their nontransgenic littermates. This challenge was lethal to >90% of all control mice (56 of 62 nontransgenic littermates died), as shown in Table 2. In contrast, the survival data (Table 2) demonstrate that three of the transgenic mouse lines showed remarkable resistance to PRV infection by means of mucosal inoculation (survival rates of 78% (18/23), 90% (9/10), and 100% (9/9) for lines 22, 37, and 45, respectively). In other transgenic mouse lines, a lower but still significant protection was observed: 50% (9/18), 61% (11/18), and 65% (11/17) of the animals from lines 6, 32, and 33 survived, respectively (Table 2). The same intranasal challenge with 10 LD_{50} of PRV strain Chiba-03, a new field isolate, was performed by using transgenic mice of line 37 and their nontransgenic littermates, with identical results: 100% (12/12) of transgenic mice survived, and 9 of 10 nontransgenic littermates died, as observed for the Japanese reference, strain YS-81 (Table 2).

Expression of PHveCIg in nasal stratified squamous epithelium (Fig. 2*A*) and pseudostratified columnar respiratory epithelium (Fig. 2*C*) of transgenic mice (line 22) and control littermates was examined by immunohistochemical staining by using anti-human IgG antibodies. Specific staining was noted on the surface of alveolar and bronchiolar epithelium (Fig. 2*E*). PHveCIg staining tended to be located in the periphery of neurons in brainstem and trigeminal ganglia (data not shown). No staining was detected in any organs from nontransgenic littermates (Fig. 2).

To test whether mice were latently infected with PRV, PCR analysis was performed to detect PRV DNA in the trigeminal ganglia of the surviving mice. Because lethal challenge with the virulent strain YS-81 does not lead to latency in control mice but to death, positive control for this test was provided by detecting PRV DNA in the trigeminal ganglia of control mice infected with the attenuated strain Begonia [250 plaque-forming units (the same no. of plaque-forming units as 10 LD_{50} of YS-81 strain)]. As shown in Fig. 3*A*, PRV DNA was not detected in the surviving transgenic mice (line 22) after intranasal inoculation with 10 LD_{50} of YS-81 strain. However, PRV DNA was detected in all of eight nontransgenic controls after intranasal inoculation with Begonia strain (Fig. 3*C*). Furthermore, no specific antibodies to PRV were found in the surviving transgenic mice by using an ELISA test (data not shown).

We also checked how PHveCIg expression would protect against latent infections observed with the attenuated PRV strain. Neither transgenic or nontransgenic littermate mice showed any symptoms or died, because Begonia strain is attenuated. PRV DNA was detected in two of eight transgenic mice inoculated with Begonia strain (Fig. 3*B*) and in all nontransgenic littermates (Fig. 3*C*). The 25% of latently infected transgenic animals is about the same as the 22% of transgenic animals that succumbed to the virulent strain (Table 2; intranasal challenge with 10 LD_{50} PRV strain YS-81 for transgenic mouse line 22).

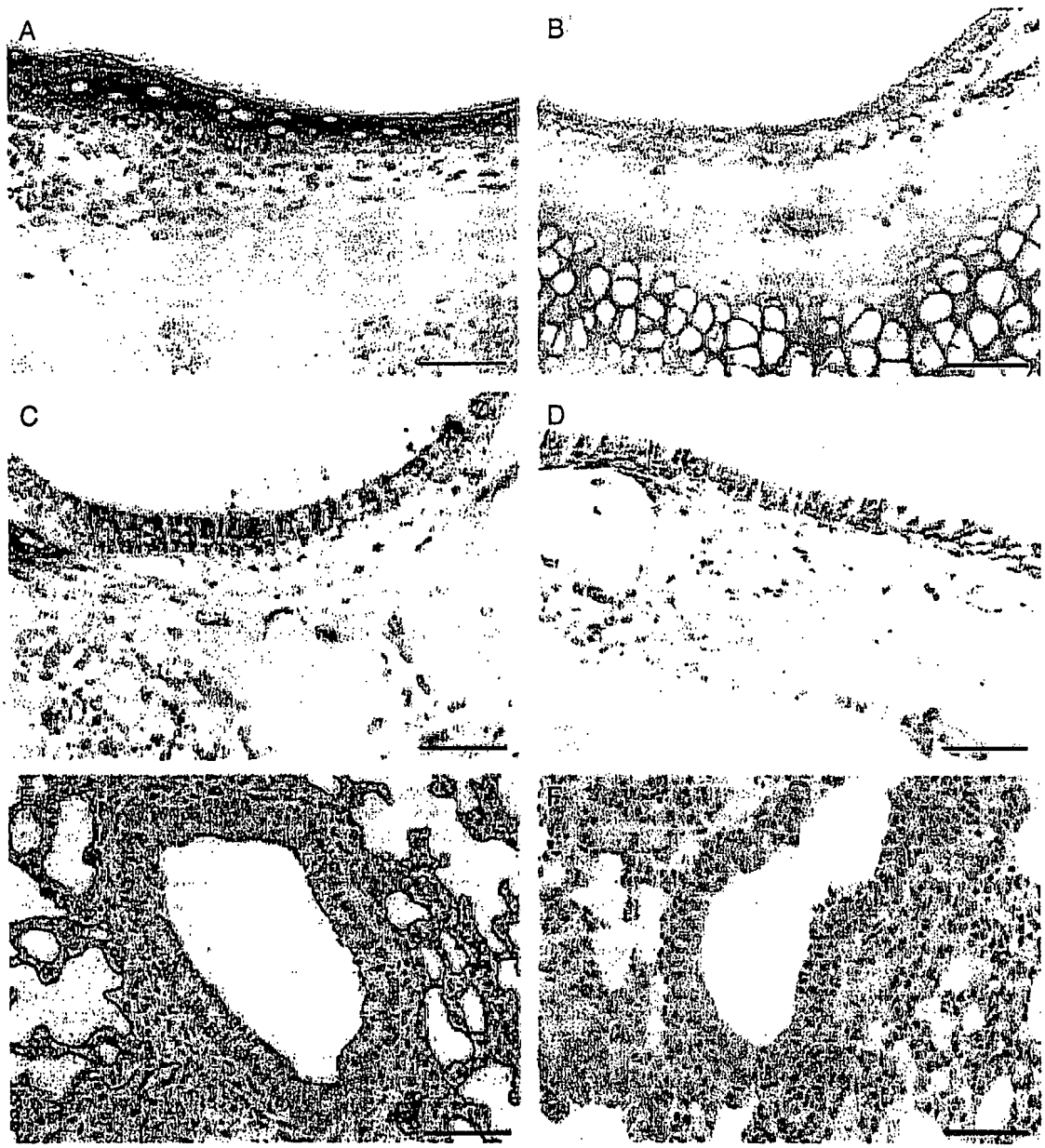

Fig. 2. Immunohistochemical staining of nasal mucosa and lung with anti-human IgG antibody of transgenic (*A*, *C*, and *E*) and nontransgenic (*B*, *D*, and *F*) littermate mice of line 22. (Bar, 50 μm.) (*A* and *B*) Nasal squamous epithelium. (*C* and *D*) Nasal respiratory epithelium. (*E* and *F*) Lung alveolar and bronchiolar epitheliums.

Specific antibodies to PRV were detected in the latently infected transgenic and nontransgenic littermate mice by using an ELISA test (data not shown). Taken together, these findings demonstrate that transgenic mice that survived after i.p. and intranasal challenge with YS-81 virulent strain were not infected, suggesting that expression of PHveCIg protects transgenic mice against PRV primary infection and not only against disease symptoms.

Inhibition of PRV Infection in Cultured Cells. To assess whether cells isolated from the transgenic mice would reflect the *in vivo* resistance to PRV infection, primary cultured embryonic fibroblasts from transgenic and control mice were infected with PRV. Embryonic fibroblasts were prepared from embryos of heterozygous transgenic (line 22) and nontransgenic parents. PHveCIg expression in the fibroblasts from the transgenic mouse was confirmed by Western blot analysis and indirect immunofluorescence assay, but was not found in the control fibroblasts (Fig. 4 *A* and *B*). Immunofluorescent staining was mainly observed in the cytoplasm (Fig. 4*B*) but was slightly detectable on the cell surface (data not shown). The transgenic and nontransgenic fibroblasts were washed twice with DMEM just before the viral inoculation to remove secreted PHveCIg in the medium and

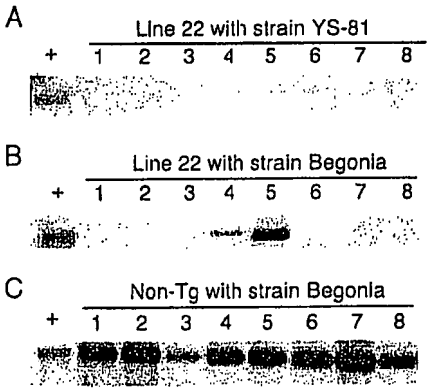

Fig. 3. Detection of PRV DNA in the trigeminal ganglia of mice after intranasal inoculation of PRV. Shown are transgenic mice (line 22) inoculated with PRV strain YS-81 (*A*) or Begonia (*B*) and nontransgenic (non-Tg) littermate inoculated with strain Begonia (*C*). The positive control (+) is a DNA extracted from PRV-infected Vero cells. The number of each lane corresponds to the mouse number tested in each experiment.

infected with PRV. In the dishes containing the transgenic fibroblasts, the number of plaques was markedly lower than in those containing the control fibroblasts 2 days after infection (Fig. 4*C*). The plaque sizes were also definitely smaller than those observed in the control fibroblasts (Fig. 4*D*). These results confirm that isolated cells from transgenic animals expressing PHveCIg are less sensitive to PRV infection than corresponding wild-type cells, even when the secreted PHveCIg molecules, which have neutralizing activity, were washed away.

Discussion

The animals described expressing PHveCIg showed a high resistance to PRV challenge by means of both i.p. and intranasal

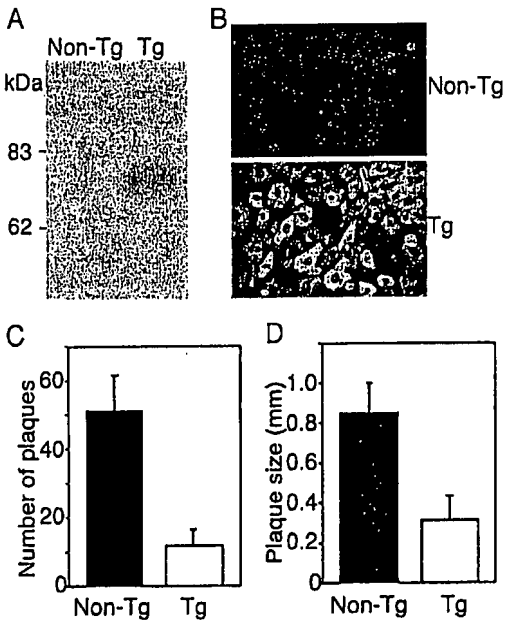

Fig. 4. Resistance to PRV infection of embryonic fibroblasts prepared from transgenic (Tg) and nontransgenic mice. (*A*) Western blot analysis of cell extracts. (*B*) Immunofluorescent staining of fibroblasts. (*C*) Number of plaques of infected fibroblasts. (*D*) Plaque size of infected fibroblasts.

routes. It is especially noteworthy that protection against PRV entry in the sites of primary infection was observed in the transgenic mice after intranasal inoculation of PRV. The transgenic embryonic fibroblasts expressing PHveCIg showed marked resistance to PRV infection. PRV DNA in the trigeminal ganglia was not detected in the surviving transgenic mice inoculated with PRV strain YS-81 by means of the intranasal route. In the transgenic nasal mucosa and respiratory tract, expression of PHveCIg was confirmed by immunohistochemical staining. These findings suggest that the epithelial cells of the nasal mucosa and respiratory tract expressing PHveCIg are also resistant to PRV infection. Transgenic mouse line 37, expressing only 5 μg/ml PHveCIg in the serum, showed significant resistance to PRV infection by means of intranasal route, indicating that such a low level of expression of the transgene is sufficient to inhibit the virus entry into cells and that circulating PHveCIg may not play an important role in the resistance to PRV infection. There are several possible effects of PHveCIg on the suppression of virus replication, as seen in transgenic primary fibroblasts. Firstly, cell-bound PHveCIg may inhibit the virus entry into fibroblasts as described previously (23). Secondly, intracellular PHveCIg may bind to newly synthesized glycoprotein D and inhibit secondary infections as intrabodies (32, 33). Thirdly, secreted PHveCIg may inhibit secondary infections of the fibroblasts, neutralizing free virus released in the first round of infection. Fourthly, cell-bound and intracellular PHveCIg may inhibit secondary infection in the fibroblasts mediated by cell-to-cell spread. These possible effects of PHveCIg, expressed in the nasal mucosa and respiratory tract, may account for the protection against intranasal PRV infection.

Nectin-1 is a known component of intercellular junctions and plays a major role in several cell functions, such as morphogenesis, differentiation, proliferation, and migration, and in the molecular mechanisms that underlie junctional disorders associated diseases. Thus, the issue of possible adverse effects needs to be addressed carefully. However, detailed histological examination of main tissues from 7-week-old transgenic mice did not reveal any differences in cell morphology induced by PHveCIg expression. Functional neuronal disorders were not suggested because no differences were observed between transgenic and control mice in the rotarod test. Transgenic mice developed normally with normal body weights and litter sizes. The smaller litter size in line 45 and the slightly lower body weights in line 6 may be due to insertional mutation of the transgene, because similar findings were not observed in other lines. Nonetheless, these effects were not correlated to higher expression levels. Primary structure of nectin-1 is well conserved across mammalian species, including mouse and pig, in which they share 95% of amino acid residues in common regarding their extracellular domain (24). In a different context, a soluble form of murine HVEM expressed in transgenic mice enhanced resistance to HSV-1 infection (17). In those transgenic mice, no gross abnormality was observed, and overexpression of a homologous form of other α-herpesvirus receptors did not produce side effects expected on the immune system. HVEM is a member of the tumor necrosis factor receptor family (12) and plays an important role on the immune systems. Taken together, these data suggest that overexpressing a soluble form of homologous nectin-1, such as PHveCIg described here in pigs, may not raise host safety issues.

Farm animals that are effectively protected against infectious disease through the use of transgenes are still concepts only, although several cellular and molecular mechanisms can theoretically be used to block the infection of cells or living organisms. Although the possibility of disease resistance in transgenic animals has been envisioned for many years, few, if any, convincing models have been validated by using *in vivo* challenges. Transgenic mice secreting coronavirus neutralizing antibodies in

their milk were reported (34–36). An example of transgene-mediated lactogenic immunity *in vivo* was provided by this approach (36). A recent review (37) suggests a coronavirus receptor knockout strategy but also mentions potential side effects. The same report underlines the wide range of viral pathogens that could be targeted by using RNA interference-mediated knockdown of viral genes. However, *in vivo* demonstration of efficiency for any of these strategies is still to come. Here, we showed that a transgene could efficiently protect animals against a major viral infectious disease by expressing a soluble form of a viral receptor.

Although animal breeding is continuously demonstrating its ability to improve production traits, it does little to improve animal health and disease resistance. The present work proves the efficiency of a previously envisaged alternative strategy by building on acquired knowledge of infection pathogeny and hitting on critical components of known host pathogen interactions to achieve a practical genetic immunization.

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